Studi Trent. Sci. Nat., 87 (2010): 77-82 ISSN 2035-7699

Assessment of genetic differentiation in Italian populations of *Austropotamobius pallipes* species complex: taxonomic and management implications

Stefania CHIESA[1*], Milena MALDINI[2], Massimiliano SCALICI[1], Francesco NONNIS MARZANO[2] & Giancarlo GIBERTINI[1]

[1] Department of Biology, Università degli Studi "Roma Tre", V.le G. Marconi 446, 00146 Roma, Italy
[2] Department of Evolutive and Functional Biology, Università degli Studi di Parma, V.le G. P. Usberti 11, 43100 Parma, Italy
[*] Corresponding author e-mail: *chiesa@uniroma3.it*

SUMMARY - *Assessment of genetic differentiation in Italian populations of* Austropotamobius pallipes *species complex: taxonomic and management implications - Austropotamobius pallipes* (Lereboullet, 1858) has been placed through several revisions, due to its high intraspecific variability: until now, two distinct species have been described in Italy, *A. pallipes* and *A. italicus*, the latter divided into four subspecies. Despite these taxonomic uncertainties, *A. pallipes* is still considered the only native freshwater crayfish in Italy, suffering a progressive decline of its populations during the latest decades, and now included in the Red List of the IUCN, in Annex III of Berna's Convention, and in Annex II and V of EU Habitats Directive (92/43/EEC). Therefore, the aim of the present study was to investigate the genetic differentiation of Italian populations of the *A. pallipes* complex, using AFLP markers. AFLP data elaboration was preceded by the characterisation of mitochondrial *16S* rDNA and *COI* haplotypes in two different reference populations, belonging to either *A. italicus* or *A. pallipes*, to verify their correct taxonomic identification as "true species". The corresponding AFLP profiles were considered as reference data for comparison with all other populations. Two hundred specimens were analysed using five *Eco*RI and *Taq*I primer combinations. More than 400 polymorphic bands were scored to generate binary data matrices (0 = electrophoretic band absence, 1 = presence) to assess within and between population differences and to suggest biogeographic considerations.

RIASSUNTO - *Indagine genetica sul complesso di specie* Austropotamobius pallipes *in Italia: valutazioni sistematiche ed implicazioni gestionali* - L'alto grado di variabilità intraspecifica ha portato a numerose revisioni tassonomiche del complesso di specie *Austropotamobius pallipes* (Lereboullet, 1858): alcuni autori identificano in Italia due specie diverse, *A. pallipes* ed *A. italicus*, con quattro sottospecie appartenenti a quest'ultima. A livello normativo *A. pallipes* è considerata l'unica specie italiana di gambero di fiume, in costante rarefazione, inclusa nella "Lista Rossa" IUCN delle specie minacciate, nell'Allegato III - Convenzione di Berna e negli Allegati II e V - Direttiva Habitat 92/43/CEE. E' stata avviata una ricerca con lo scopo di caratterizzare alcune popolazioni italiane del complesso *A. pallipes* attraverso l'impiego di marcatori AFLP. L'analisi dei profili AFLP dei singoli esemplari è stata preceduta dalla determinazione degli aplotipi mitocondriali *16S* e *COI* in un congruo numero di campioni di *A. italicus* e di *A. pallipes*, per verificare la loro appartenenza a "buone specie", e per l'attribuzione di profili fingerprinting specifici ai due gruppi sistematici. I corrispondenti pattern AFLP sono stati utilizzati come riferimento nel confronto tra tutte le altre popolazioni analizzate. Sono state utilizzate cinque combinazioni di *primer Eco*RI e *Taq*I in 200 esemplari. L'analisi di oltre 400 marcatori polimorfici, organizzati in matrici di dati binari (0 = assenza di banda elettroforetica, 1 = presenza) ha permesso di individuare differenze entro e tra popolazioni, consentendo di formulare considerazioni biogeografiche.

Key words: freshwater crayfish, *Austropotamobius pallipes*, conservation, genetic differentiation, populations, AFLP markers
Parole chiave: gambero di fiume, *Austropotamobius pallipes*, conservazione, variabilità genetica, popolazioni, marcatori AFLP

1. INTRODUCTION

Austropotamobius pallipes (Lereboullet, 1858) (Decapoda, Astacidae), the only native freshwater crayfish in Italy, plays an important role in the trophic web of freshwater ecosystems (Scalici *et al*. 2004), and it can be considered an useful bioindicator of water quality (Scalici & Gibertini 2005). Once widely distributed in European countries, from Italy to the United Kingdom and former Yugoslavia (Fratini *et al*. 2005), the white-clawed crayfish in the latest decades was affected by a progressive decline of its populations, as a consequence of many different factors: degradation and loss of freshwater habitats, fishing exploitation and introduction of exotic crayfish species, which may act as competitors and vectors of crayfish plague *Aphanomyces astaci* Shikora (Holdich & Reeve 1991). This dramatic situation increased the attention on the conservation status of *A. pallipes*: actually, it is considered "vulnerable" on the Red List of the IUCN, and is included in Annex II and V of EU Habitats Directive (92/43/EEC) and in Annex III of Berna's Convention.

In conservation programs many erroneous decisions may result if the taxonomic status of a species or a population is not correctly assigned (Frankman *et al*. 2002). *A. pallipes* has been placed through several revisions, due

to its high morphological and genetic variability (Fratini *et al*. 2005). Many different studies have been carried out in Europe to resolve this controversy, using varying criteria to classify the different groups. Based on a limited number of morphological and meristic parameters, Bott (1950) recognised a single species, *A. pallipes*, and three subspecies; Karaman (1962) distinguished the two species *A. pallipes* and *A. italicus*, the latter divided into three subspecies, while Bott (1972) reported the existence of endemic species *A. berndhauseri* in Swizerland. In recent decades, the improvement of molecular techniques provided more information on allozyme (Santucci *et al*. 1997) and genetic diversity, leading to a re-examination based on *16S* rDNA gene by Grandjean *et al*. (2002). According to these results, two species exist, *A. pallipes* (France and British Isles) and *A. italicus*, this one divided into three subspecies: *A. i. italicus* (Italy, France, Spain), *A. i. carsicus* (France and Slovenia) and *A. i. carinthiacus* (Austria, and Swizerland). Recently, Fratini *et al*. (2005) using *16S* mtDNA sequencing of Italian populations, suggested the occurrence of both distinct species in Italy, *A. pallipes* and *A. italicus*, the former confined to North-Western regions, the latter distributed across the entire peninsula and divided into four subspecies: the three mentioned above, and the newly proposed *A. i. meridionalis* in the Central and Southern regions. The two different species overlap only in the Ligurian Apennine.

Zaccara *et al*. (2005) investigated the diversity of *A. italicus* also using the *COI* mitochondrial gene for the populations of Po River drainage basin, but in a larger study Trontelj *et al*. (2005), used the *COI* gene sequences to investigate phylogenetic and phylogeographic relationships for the entire European freshwater crayfish genus *Austropotamobius*. They recognised the two traditional taxa *Austropotamobius torrentium* (Schrank, 1803) and *A. pallipes*, but the split of the former taxon into *A. pallipes* and *A. italicus* could not be deduced from phylogenetic trees (Trontelj *et al*. 2005). As a consequence, systematic uncertainties still remain. Therefore, the aim of the present study was to investigate the genetic differentiation of Italian populations of the *A. pallipes* complex, using both mitocondrial and nuclear markers, with a methodological approach based on *16S* rDNA and *COI* genes sequencing and AFLP polymorphisms.

2. STUDY AREA

We collected 199 specimens of the white-clawed crayfish, caught both by hand and nets, from 35 Italian populations. Sampling sites (Table 1) were chosen from the Alps to Southern Apennines to cover a wide portion of the Italian distribution of the species. In addition, we collected 3 individuals of *A. torrentium* from Hungary to be used as outgroup for the mitochondrial and AFLP analyses.

3. METHODS

High molecular weight genomic DNAs were extracted and purified from ethanol-fixed muscle tissue stored at –20 °C. Muscle tissues were collected from fragments of pleiopods and chelae taken from live samples, without sacrifice of animals. DNA was extracted either according to the clas-

Tab. 1 - Details of examined populations: N = population number, S. SITES = sampling sites (Provinces), POP. = population label, SPEC. N = number of specimen analyzed for each population.
Tab. 1 - Popolazioni esaminate: N = numero della popolazione, S. SITES = stazioni di campionamento (provincia), POP. = sigla identificativa della popolazione, SPEC. N = numero di esemplari analizzati per ciascuna popolazione.

N	S. SITES	POP.	SPEC.N
1	Fosso Ariana (Rieti)	A	4
2	Fosso Argentina (Terni)	AR	2
3	Bussento (Salerno)	B	4
4	Bragamonti (Cuneo)	BR	3
5	Bussentino (Salerno)	BU	7
6	Torrente Oxentina (Imperia)	C	3
7	Castagno d'Andrea (Firenze)	CD	11
8	Rio Ceppeta (Arezzo)	CEP	14
9	Fontanile (Cremona)	CR	5
10	Fosso Duranna (Roma)	D	3
11	Lago Pranda (Reggio Emilia)	LP	5
12	Il Mulino (Firenze)	M	1
13	Fosso Doglio (Perugia)	MC	5
14	Torrente Nenno (Genova)	N	10
15	Fosso Olpeta (Viterbo)	OL	3
16	Fosso dei Pantani (Viterbo)	P	4
17	Torrente Licenza (Roma)	PE	12
18	Rio Rialasco (Genova)	R	3
19	Rio Canvella (Prato)	RC	7
20	Rio Gamberi (Verbania)	RG	4
21	Torrente Chiusola (La Spezia)	TC	2
22	Roggia di Terlago (Trento)	TR	5
23	Risorgive del Ticino (Novara)	TIC	4
24	Torrente Stirone (Parma)	STIRONE	4
25	Udine	UD	7
26	Fosso Vetrina (Chieti)	V	3
27	Valle Peso (Cuneo)	VP	3
28	Torrente Zena (Bologna)	ZE	2
29	Pistoia	PIS	10
30	Rio Valturcana (Belluno)	VTU	20
31	Torrente Verde (Chieti)	CH	3
32	Chieti	S	2
33	Arandolo (Cuneo)	AN	5
34	Torrente Rossana (Cuneo)	ROS	7
35	Villa D'Agri (Potenza)	BAS	12
36	Ungheria	A. torr.	3

sical SDS-proteinase K and phenol-chloroform technique described by Moore (1999) or alternatively by means of the Aquapure genomic DNA kit (Biorad). DNA quality was assessed by means of electropohresis on 1% agarose gel in TAE buffer.

Characterisation of both *16S* e *COI* mitochondrial haplotypes was carried out in two different reference populations, belonging to either *A. pallipes* (Nenno-"N") or *A. italicus* (Pistoia-"PIS") sensu Grandjean *et al.* (2002) and Fratini *et al.* (2005) to verify their correct taxonomic identification as "true species" for a total of 20 specimens. The corresponding AFLP profiles were considered as reference data for comparison with all other populations.

We used the primers HCO 2198 and LCO 1490 (Folmer *et al.* 1994; Trontelj *et al.* 2005; Dawnay *et al.* 2007) to amplify a fragment of the *COI* gene, by applying 40 cycles of 30s at 95°C, 45 s at 45°C, and 1 min at 72°C, after an initial 10 min denaturation step at 95°C.

Primers *16S*ar and *16S*br (Fratini *et al.* 2005) were used to amplify a fragment of the *16S* gene with the following conditions: 35 cycles of 30s at 95°C, 30 s at 48°C, and 45s at 72°C, after an initial 5 min denaturation step at 95°C. PCR products were purified by eluition from a 2.5% agarose gel, then precipitated with 3 volumes of 100% ethanol, and washed with 70% ethanol. Both genes were sequenced in the forward and reverse directions with CEQTM DTCS-Quick Start Kit (Beckman Coulter) on "CEQ™ 8000 DNA Analysis System" (Beckman Coulter).

The sequences were compared with data available in genomic databases using a BLAST procedure and multiple alignments were produced using the software Clustal X and Sequencher, verifying the correctness of the aligment at single nucleotide.

The Neighbour-Joining trees were obtained from the *COI* and *16S* sequences using Phylip software 3.6 and visualised by Treeview sofware (version 1.6).

AFLPs profiles were obtained following the protocol by Maldini *et al.* (2006), using 5 primers combinations: E32-T33 (eAAC-tAAG); E33-T32 (eAAG-tAAC); E32-T32 (eAAC-tAAC); E40-T37 (eAGC-tACG); E33-T37 (eAAG-tACG). A 1.7 µl volume of PCR product and 0.3 µl of DNA internal size standards (CEQ DNA Size standard-600 Beckman-Coulter) were added to 40 µl of deionized formamide (J.T. Baker, Phillipsburg, NJ). Samples were then loaded into the "CEQ™ 8000 DNA Analysis System" (Beckman Coulter). Running conditions for capillary electrophoresis, raw data elaboration and reproducibility are reported in Papa *et al.* (2005). Analysed data were exported to Genographer software (Vers.1.6.0, Benham J.J., Montana State University 2001), for single specimen comparison and scoring. Genographer software allows the construction of a virtual gel with bands shaped on the base of peak height, resolution and mobility and permits a thorough analysis of single fragments. Presence or absence of fragments in each individual were scored as 1 or 0, respectively. A binary data matrix was then created and analyzed with the sofware Genetix which allows a multivariate statistical analysis of AFLP data.

4. RESULTS

A region of 600 bp was amplified and sequenced within the *16S* gene sequence, whilst 650 bp were obtained

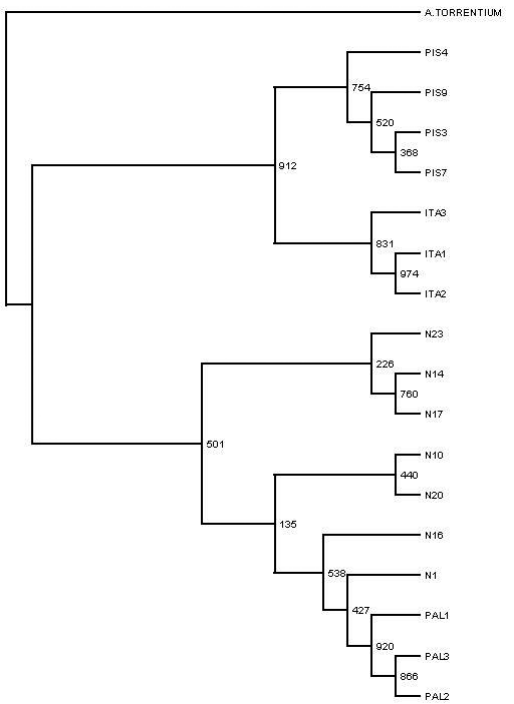

Fig. 1a - Neighbour-Joining tree inferred by the *16S* gene sequences analysis, with bootstrap values.
Fig. 1a - Albero filogenetico ottenuto con il metodo di Neighbour-Joining dall'analisi delle sequenze del gene mitocondriale 16S, con i rispettivi valori di bootstrap.

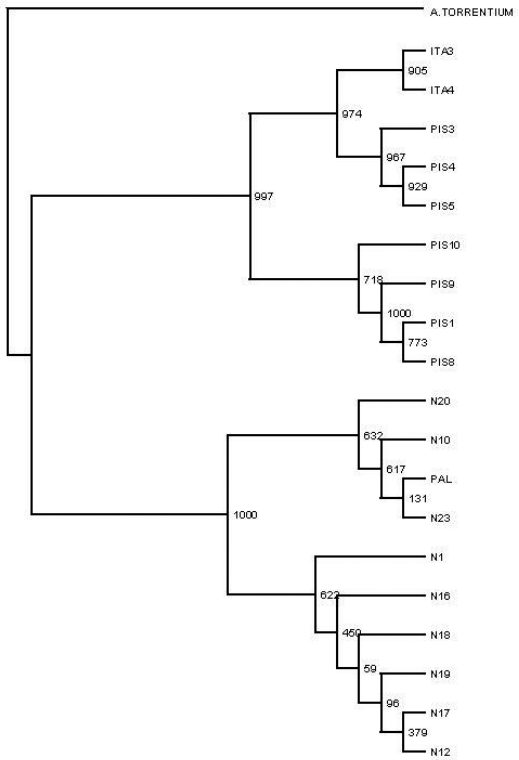

Fig. 1b - Neighbour-Joining tree inferred by the *COI* gene sequences analysis, with bootstrap values.
Fig. 1b - Albero filogenetico ottenuto con il metodo di Neighbour-Joining dall'analisi delle sequenze del gene mitocondriale COI, con i rispettivi valori di bootstrap.

Tab. 2 - Mitochondrial *16S* and *COI* genes sequences from GenBank included in the analysis.
Tab. 2 - Sequenze dei geni mitocondriali 16S e COI ottenute da GenBank ed incluse nell'analisi.

mtDNA gene	taxon	sequence label	GenBank accession number and original source
16S	*A. pallipes*	PAL1	AY521285 (Zaccara *et al*., 2004)
16S	*A. pallipes*	PAL2	AY611203 (Fratini *et al*., 2005)
16S	*A. pallipes*	PAL3	AY521287 (Zaccara *et al*., 2004)
16S	*A. italicus*	ITA1	AY611189 (Fratini *et al*., 2005)
16S	*A. italicus*	ITA2	AY611187 (Fratini *et al*., 2005)
16S	*A. italicus*	ITA3	AY611185 (Fratini *et al*., 2005)
16S	*A. torrentium*	*A. torrentium*	AM181348 (Schubart & Huber, 2006)
COI	*A. pallipes*	PAL	AY667115 (Trontelj *et al*., 2004)
COI	*A. italicus*	ITA3	AY121114 (Iaconelli & Cianchi, 2002)
COI	*A. italicus*	ITA4	AY121115 (Iaconelli & Cianchi, 2002)

Tab. 3 - Monomorphic, polymorphic and total loci scored by AFLPs analysis.
Tab. 3 - Loci monomorfici, polimorfici e totali ottenuti attraverso l'analisi dei marcatori AFLP.

PRIMER COMBINATION	MONOMORPHIC LOCI	POLYMORPHIC LOCI	TOTAL	% POLYMORPHISM
eAGC tACG	12	86	98	88
eAAG tACG	9	104	113	92
eAAG tAAC	8	86	94	91
eAAC tAAC	15	96	111	86
eAAC tAAG	12	107	119	90
TOTAL 5	56	479	535	90

from *COI* gene. Sequences were aligned with Genbank data (Table 2) to a final length of 450 bp and 400 bp for *16S* and *COI* gene respectively. Neighbour-Joining trees were obtained with high bootstrap values (Fig. 1a, 1b).

Mitochondrial haplotypes for both *16S* and *COI* genes were split into two major clades corresponding to Nenno (N) and Pistoia (PIS) specimens: Nenno's always grouped with *A. pallipes* sequences, while Pistoia's with *A. italicus* sequences. *A. torrentium* was also separated from the *A. pallipes-italicus* group.

Considering AFLPs, 1010 fingerprinting profiles were obtained from 202 suitable specimens amplified with 5 primers combinations. A total of 535 bands were scored, 56 of which were monomorphic, while 479 were polymorphic. The mean number of bands was about 107 for each primer combination. Monomorphic loci ranged from 8 to 15, and polymorphic ones ranged from 86 to 107, depending on the primer combination (Table 3). The total percentage of polymorphism was about 90%, a high but expected value using this tecnique, which returns fingerprinting profiles relative to the entire genome. Results were in accordance with what described by previous AFLP studies on crustaceans and insects (Sun *et al*. 1999, Mendelson & Shaw 2002; Salvato *et al*. 2002; Cannas *et al*. 2003; Gili *et al*. 2004; Mendelson *et al*. 2004). Considering the two main reference populations characterised by *16S* and *COI* mitochondrial sequences, Nenno corresponding to *A. pallipes* (*sensu* Grandjean *et al*. 2002) and Pistoia corresponding to *A. italicus italicus* (*sensu* Grandjean *et al*. 2002), only 42 bands out of 535 (9%) were diagnostic (i.e. always present in a group and absent in the other); conversely, the two populations shared 27% of the total bands (144 out of 535). Besides, 11% of the loci were monomorphic in all the examined individuals of *A. pallipes* complex.

Considering the 3-Dimensional FCA (Factorial Correspondence Analysis) generated by the software Genetix (Fig. 2), the congeneric outgroup *A. torrentium* is well separated, while most populations of *A. pallipes* complex are grouped together, showing a high overall variability within the entire dataset and no strong subclustering of populations. The main factor is explained by Axis 1, accounting for 15.53% of the total variance.

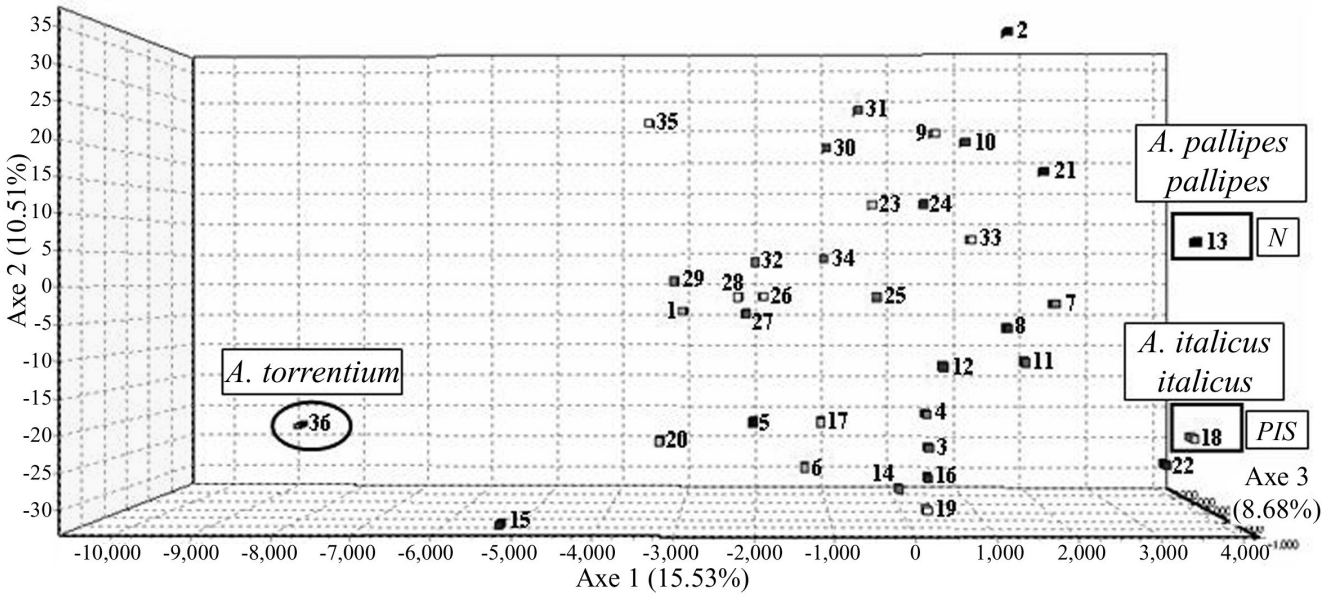

Fig. 2 - 3-dimensional Factorial Correspondence Analysis of Austrapotamobius populations (Nenno = population n°14, Pistoia = population n° 29, *A. torrentium* = population n° 36).
Fig. 2 - Analisi Fattoriale delle Corrispondenze in 3 dimensioni delle popolazioni di Austrapotamobius (Nenno = popolazione n°14, Pistoia = popolazione n° 29, A. torrentium *= popolazione n° 36).*

5. DISCUSSION

Considering the *16S* and *COI* gene sequences, two different haplogroups were observed within the *A. pallipes* complex, corresponding to the two reference populations and which could possibly match the taxa described by Grandjean *et al*. (2002). Nevertheless, the AFLP fingerprinting does not reveal significant differences among populations and suggests the presence of a unique, highly variable group. Is it possible to explain this apparent contradiction beetween mitochondrial and AFLP fingerprinting results?

AFLP markers are a powerful tool to assess genetic diversity, in particular for establishing relashionships among closely related taxa (Mendelson & Shaw 2005). They have many advantages, due to their time and cost effectiveness, replicability and resolution efficiency which are equal or superior to those of other markers (Mueller & Wolfenbarger 1999), and they generate hundreds of informative loci with a high number of polymorphisms (Papa *et al*. 2003).

In this case, more than 500 analysed loci seem to indicate that the *A. pallipes* and *A. italicus* species sensu Grandjean *et al*. (2002) are not well separated, as also observed by Trontelj *et al*. (2005). Maybe as a result of an ancient but incomplete speciation process, the present-day mitochondrial variability is structured into well defined haplogroups, but nDNA evidence suggests that the two taxa can not be considered as different and reproductively isolated species.

6. CONCLUSIONS

This study revealed that, conversely to what assessed by mitochondrial markers, in particular *16S* rDNA gene sequences, one unique species of native freshwater crayfish occurs in Italy: *A. pallipes*; analysing more than 500 loci of the entire genome with AFLP markers, we did not find evidence of a separation beetween this taxon and *A. italicus*.

To confirm this hypotesis, also the outcomes of morphometric investigations could play an important role; in fact, species-specific diagnostic morphological characters were never identified for *A. pallipes* and *A. italicus*. Geometric morphometry represents an interesting additional approach to evaluate differences between these two groups, because it can explore not only meristic or morphometric charaters, but also variation of the whole shape of a single structure. A geometric morphometric study on *A. pallipes* and *A. italicus* is currently in its final stages and the results seem to support the genetic evidence (Scalici, pers. comm.).

ACKNOWLEDGEMENTS

The authors would like to thank G. Caricato, M. De Biaggi, G. De Luise, S. Fratini, F. Gherardi, E. Marconato, T. Pagliani, S. Salvidio and M. Zanetti for their help in collecting samples in Italy, and Lazlo Forrò from the Magyar Termeszettudomanyi Muzeum of Budapest for *A. torrentium* samples.

REFERENCES

Bott R., 1950 - Die Flüssekrebse Europas (Decapoda, Astacidae). *Proceedings of the Senckenberg Naturalist Society*, 483: 1-36.

Bott R., 1972 - Besiedlungsgeschichte und Systematik der Astacieden West-Europas unter besonderer Beruchksichtigung der Schweiz. *Revue Suisse de Zoologie*, 79: 387-408.

Cannas A., Deiana A.M. & Tagliavini J., 2003 - AFLP-fingerprinting delle aragoste mediterranee *Palinurus elephas* e *P. mauritanicus*. Dati preliminari. *Biol. Mar. Medit*., 10/2: 379-382.

Dawnay N., Ogden R., McEwing R., Carvalho G.R. & Thorpe R.S., 2007 - Validation of the barcoding gene *COI* for use in forensic genetic species identification. *Forensic Sci. Int*., 173: 1-6.

Folmer O., Black M., Hoeh W., Lutz R. & Vrijehoek R., 1994 - DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol*., 5: 294-299.

Frankman R., Ballou J.D. & Briscoe D.A., 2002 - Resolving taxonomic uncertainties and defining management units. In: Frankman R., Ballou J.D. & Briscoe D.A. (eds). *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge: 365-394.

Fratini S., Zaccara S., Barbaresi S., Grandjean F., Souty-Grosset C., Crosa G. & Gherardi F., 2005 - Phylogeography of the threatened crayfish (genus *Austropotamobius*) in Italy: implications for its taxonomy and conservation. *Heredity*, 94: 108-118.

Gili M., Monaghan M.T. & Spaak P., 2004 - Amplified fragments length polymorphisms (AFLP) reveals species-specific markers in the *Daphnia galeata-hyalina* species complex. *Hydrobiologia*, 526/1: 63-71.

Grandjean F., Bouchon D. & Souty-Grosset C., 2002 - Systematics of the European endangered crayfish species *Austropotamobius pallipes* (Decapoda, Astacidae) with a re-examination of the status of *Austropotamobius berndhauseri*. *J. Crustacean Biol*., 22/3: 677-681.

Holdich D.M. & Reeve I.D., 1991 - Distribution of the freshwater crayfish in the british isles, with particular reference to crayfish plague, alien introductions and water quality. *Aquat. Conserv.,* 1: 139-158.

Karaman M.S.,1962 - Ein Beitrag zur Systematik der Astacidae. *Crustaceana*, 2: 174-191.

Maldini M., Nonnis Marzano F., González Fortes G., Papa R. & Gandolfi G., 2006 - Fish and seafood traceability based on AFLP markers: Elaboration of a species database. *Aquaculture,* 261: 487-494.

Mendelson T.C. & Shaw K.L., 2002 - Genetic and behavioral components of the cryptic species boundary between *Laupala cerasina* and *L. kohalensis* (*Orthoptera*: *Grillidae*). *Genetica*, 116: 301-310.

Mendelson T.C.& Shaw K.L., 2005 - Use of AFLP markers in surveys of arthropod diversity. *Method. Enzymol*., 395: 161-177.

Mendelson T.C., Siegel A.M. & Shaw K.L., 2004 - Testing geographic pathways of speciation in a recent island radiation. *Mol. Ecol*., 13: 3787-3796.

Moore D., 1999 - Manipulation of DNA. In: Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., Struhl K. (eds.), *Current Protocols in Molecular Biology*. John Wiley & Sons Inc., New York, 211-223:

Mueller U.G. & Wolfenbarger L.L., 1999 - AFLP genotyping and fingerprinting. *Tree*, 14/10: 389-394.

Papa R., Nonnis Marzano F., Rossi V. & Gandolfi G., 2003 - Genetic diversity and adaptability of two species of Mugilidae (Teleostei: Perciformes) of the Po river delta coastal lagoons. *Oceanol. Acta*, 26: 121-128.

Papa R., Troggio M., Ajmone-Marsan P. & Nonnis Marzano F., 2005 - An improved protocol for the production of AFLP markers in complex genomes by means of capillary electrophoresis. *J. Anim. Breed. Genet*., 122: 62-68.

Salvato P., Battisti A., Concato S., Masutti L., Patarnello T. & Zane L., 2002 - Genetic differentiation in the winter pine processionary moth (*Thaumetopoea pityocampa-wilkinsoni complex*) inferred by AFLP and mitochondrial DNA markers. *Mol. Ecol*., 11: 2435-2444.

Santucci F., Iaconelli M. & Andreani R., 1997 - Allozyme diversity of european freshwater crayfish of the genus *Austropotamobius*. *Bull. Fr. Peche Pisc*., 347: 663-676.

Scalici M., Vignoli L. & Gibertini G., 2004 - Osservazioni sulle abitudini alimentari del gambero di fiume nostrano, *Austropotamobius pallipes italicus* (Faxon). *Biol. Amb*.,18/1: 51-55.

Scalici M. & Gibertini G., 2005 - Can *Austropotamobius italicus meridionalis* be used as a monitoring instrument in central Italy? Preliminary observations. *Bull. Fr. Peche Pisc*., 376-377: 613-625.

Sun Y., Song W.Q., Zhong Y.C., Zhang R.S., Abatzopoulos T.J. & Chen R.Y., 1999 - Diversity and genetic differentiation in *Artemia* species and populations detected by AFLP markers. *International Journal of Salt Lake Research*, 8: 341-350.

Trontelj P., Machino Y. & Sket B., 2005 - Phylogenetic and phylogeographic relationships in the crayfish genus *Austropotamobius* inferred from mithocondrial *COI* gene sequences. *Mol. Phylogenet. Evol*., 34: 212-226.

Zaccara S., Stefani F. & Crosa G., 2005 - Diversity of mitochondrial DNA of the endangered white-clawed crayfish (*Austropotamobius italicus*) in the Po River catchment. *Freshwat. Biol*., 50 : 1262-1272.